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Pervanadate activation of intracellular kinases leads to tyrosine phosphorylation and shedding of syndecan-1

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Syndecan-1 is a transmembrane heparan sulphate proteoglycan that binds extracellular matrices and growth factors, making it a candidate to act between these regulatory molecules and intracellular signalling pathways. It has a highly conserved transmembrane/cytoplasmic domain that contains four conserved tyrosines. One of these is in a consensus sequence for tyrosine kinase phosphorylation. As an initial step to investigating whether or not phosphorylation of these tyrosines is part of a signal-transduction pathway, we have monitored the tyrosine phosphorylation of syndecan-1 by cytoplasmic tyrosine kinases in intact cells. Tyrosine phosphorylation of syndecan-1 is observed when NMuMG cells are treated with sodium orthovanadate or pervanadate, which have been shown to activate intracellular tyrosine kinases. Initial studies with sodium orthovanadate demonstrate a slow accumulation of phosphotyrosine on syndecan-1 over the course of several hours. Pervanadate, a more effective inhibitor of phosphatases, allows detection of

phosphotyrosine on syndecan-1 within 5 min, with peak phosphorylation seen by 15 min. Concurrently, in a second process activated by pervanadate, syndecan-1 ectodomain is cleaved and released into the culture medium. Two phosphorylated fragments of syndecan-1 of apparent sizes 6 and 8 kDa remain with the cell after shedding of the ectodomain. The 8 kDa size class appears to be a highly phosphorylated form of the 6 kDa product, as it disappears if samples are dephosphorylated. These fragments contain the C-terminus of syndecan-1 and also retain at least a portion of the transmembrane domain, suggesting that they are produced by a cell surface cleavage event. Thus pervanadate treatment of cells results in two effects of syndecan-1: (i) phosphorylation of one or more of its tyrosines via the action of a cytoplasmic kinase(s) and (ii) cleavage and release of the ectodomain into the medium, producing a C-terminal fragment containing the transmembrane/cytoplasmic domain.

INTRODUCTION

Regulation of cellular function by a combination of growth factors and extracellular matrix proteins is a subject of growing interest. Syndecan-1 is a heparan sulphate proteoglycan that binds growth factors and extracellular matrix components via its heparan sulphate chains and is thus a candidate for helping to convey signals from the extracellular matrix to the cytoplasm [1,2]. Syndecan-1 binds extracellular matrix proteins such as fibronectin, thrombospondin, collagen types I, III, V and tenascin [3–6]. It also binds ‘heparin-binding’ growth factors such as members of the fibroblast growth factor family, first shown by Kiefer et al. [7]. The binding of fibroblast growth factor by heparan sulphate proteoglycans is an important step in the growth factor’s biological action [8–10]. In addition to binding its extracellular ligands, it is proposed that syndecan-1 attaches to the actin cytoskeleton through its cytoplasmic domain [11,12].

Syndecan-1 is a member of the syndecan family that consists of four proteoglycans expressed in vertebrate cells (syndecans 1–4; see [1]). The defining characteristic of the syndecan family is a highly conserved transmembrane and cytoplasmic domain which contains four conserved tyrosines and a variable number of serines/threonines that may serve as phosphorylation sites.

Phosphorylation is an important means of propagating intracellular signals. Many growth factor receptors have tyrosine kinase domains that are activated in response to binding their respective ligands (for recent reviews see [13,14]). Activation

leads to phosphorylation of the receptors themselves and initiation of a cascade of phosphorylation events that activate or deactivate a wide variety of other kinases and regulatory molecules, ultimately resulting in changes in cellular behaviour [15]. Other growth factor receptors lack inherent kinase activity, but associate with intracellular kinases when bound to an extracellular ligand [13,14]. In a similar fashion, the extracellular matrix can also generate such intracellular signals by binding and triggering receptors (e.g. integrins) that are not kinases themselves, but localize and activate kinases such as focal adhesion kinase (pp125^{FAK}) and *Src* in response to ligand binding [16–19]. The report of syndecan-4 localization in focal adhesions suggests the potential involvement of syndecans in adhesion-induced signalling [20]. Recent reports demonstrate a direct mechanism for the activation of *ras* by integrin-mediated signals [21]. In many cases, such signals are initiated by the phosphorylation of proteins on tyrosines, which interact with regulatory molecules containing SH2 phosphotyrosine-binding domains (reviewed in [22,23]). These molecules in turn often contain enzymic activities capable of further propagating a signal.

Although syndecan-1 itself does not have a kinase domain that could initiate a phosphorylation cascade, the four conserved tyrosines of its cytoplasmic domain have the potential to transduce a signal if phosphorylated (see Figure 1A). One of the tyrosines is contained in the sequence motif KKDEGSY, which is exactly conserved in all vertebrate syndecans and in the *Drosophila* syndecan [24] and fits the consensus sequence for

Abbreviations used: DMEM, Dulbecco’s modified Eagle’s medium; NEM, *N*-ethylmaleimide; PLC- γ , phospholipase C- γ ; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C.

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tyrosine kinase phosphorylation [25,26]. It has also been proposed that phosphorylation of the cytoplasmic domain may serve to regulate the association of the protein with the cytoskeleton [25]. The role of the proteoglycan in dictating cell morphology may also be regulated via cleavage of the extracellular domain of the syndecan, thus divorcing the cell from its matrix anchorage. A dibasic amino acid sequence present in syndecans-1–3 has been proposed as a likely target for a cell surface protease that would carry out this function [25–27]. The location of the cleavage site is questioned, however, by the more recent cloning of the *Drosophila* syndecan, which is shed by cultured cells despite lacking the dibasic sequence altogether [24].

To investigate the potential phosphorylation of syndecan-1 by cellular tyrosine kinases, we have utilized pervanadate. Pervanadate is a powerful phosphatase inhibitor that leads to the accumulation of phosphorylated proteins and the activation of kinases that are normally retained in inactive forms by dephosphorylation [28,29]. Pervanadate activation of the phosphorylation process in NMuMG epithelial cells and syndecan-1-transfected Raji lymphoid cells results in the rapid phosphorylation of tyrosine in the syndecan-1 cytoplasmic domain. Secondly, the extracellular domain of syndecan-1 is shed due to cleavage at the cell surface, presumably via activation of a membrane protease via an intracellular signalling mechanism.

MATERIALS AND METHODS

Cell culture and treatments

Normal murine mammary gland (NMuMG) cells were cultured to 70% confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated calf serum (Hyclone Laboratories, Logan, UT, U.S.A.), 100 units/ml penicillin, 100 µg/ml streptomycin and 10 µg/ml insulin [30]. Syndecan-expressing human Raji lymphoid cells [31] were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated calf serum, and penicillin/streptomycin.

Generation of syndecan-1-specific antibody S1CD

A ten-amino acid peptide (KPTKQEEFYA) corresponding to the C-terminus of syndecan-1 was synthesized by Multiple Peptide Systems (San Diego, CA, U.S.A.). An additional cysteine residue was added to the N-terminus of the peptide to facilitate its coupling to keyhole limpet haemocyanin, used as a carrier protein for the immunization of rabbits. Peptide-specific antibodies were purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by chromatography on peptide coupled to SulfoLink gel (Pierce, Rockford, IL, U.S.A.). Bound antibodies were eluted with 0.1 M glycine (pH 2.8) and immediately neutralized with 1 M Tris/HCl, pH 9.5. Specificity of the antibodies was determined by immunoblotting lysates of NMuMG, Raji parental and Raji syndecan-1-transfected cells; the antibody recognizes the decorated proteoglycan, the core protein after removal of the glycosaminoglycan chains, and a 12 kDa peptide consisting of the transmembrane and cytoplasmic domains of the core protein, which is maintained with the cell lysate after trypsin cleavage. Pre-adsorption of antibody with the immunization peptide results in the loss of all staining. This antibody is designated S1CD and has also been used elsewhere [31].

Immunoprecipitation

Cells (1×10^7 /ml) were lysed in extraction buffer (PBS containing 5% Triton X-100, 0.1% SDS, 1 mM PMSF, 1 mM *N*-ethyl-

maleimide (NEM), 0.1 mM sodium orthovanadate and 3 mM H_2O_2) at 4 °C for 10 min. Insoluble cell debris was removed by centrifugation at 5000 *g* for 5 min and then 3×10^7 cell equivalents of lysate were incubated with 7.5 µg/ml S1CD and 50 µl of protein A-agarose for 2 h at 4 °C. Pellets were washed four times with PBS containing 1 mM PMSF, 1 mM NEM, 0.1 mM sodium orthovanadate and 3 mM H_2O_2 , then extracted in sample buffer (190 mM Tris, pH 8.45, containing 0.5% glycerol, 1% SDS, 1 mg/ml Bromophenol Blue, 1 mM PMSF, 1 mM NEM, 0.1 mM sodium orthovanadate and 3 mM H_2O_2).

Phosphatase treatment

Cells were lysed in extraction buffer excluding the sodium orthovanadate and H_2O_2 and then immunoprecipitated as above. The pellets were washed 3 times with PBS containing 1 mM PMSF and 1 mM NEM and then with 25 mM Mes, pH 6, containing 1 mM PMSF and 1 mM NEM. Pellets were heated on a boiling water bath for 5 min, cooled to 32 °C and potato acid phosphatase (Calbiochem, San Diego, CA, U.S.A.) in 25 Mes, pH 6, was added to a final concentration of 30 units/ml. Samples were incubated for 2 h at 37 °C, then extracted in sample buffer.

Immunoblotting

Samples were heated to 95 °C for 5 min and electrophoresed in a Tris/Tricine SDS/PAGE buffer system, [32] on a 5/16% polyacrylamide step gel and electrophoretically transferred to cationic membrane (Zeta-probe, Bio-Rad, Rockville Center, NY, U.S.A.) for 14 h at 50 V in 12.5 mM Trizma base and 100 mM glycine. The membrane was fixed with 0.05% glutaraldehyde in PBS for 30 min at room temperature, rinsed, and incubated first in blocking solution (PBS containing 5% non-fat dry milk, 0.5% Nonidet P40 and 0.3% Tween 20) for 2 h at 37 °C and then in PBS containing 50 µg/ml heparin (Sigma, St. Louis, MO, U.S.A.) and 0.1% Nonidet P40 for 30 min at 37 °C. The heparin aids in blocking the cationic sites and reducing background. Blots were then incubated with 1:1000 dilution of anti-phosphotyrosine antibody (PY-20; ICN Biomedicals) for 2 h at room temperature in PBS containing 1% BSA and 0.02% NaN_3 , then washed five times with PBS containing 0.1% Nonidet P40. Blots were further incubated with biotin-conjugated anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) in PBS containing 0.1% Nonidet P40 for 30 min, then washed and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin (Amersham Corp., Arlington Heights, IL, U.S.A.) and horseradish peroxidase streptavidin (Amersham Corp.) in PBS containing 0.1% Nonidet P40 for 45 min, then finally washed and detected with an enhanced chemiluminescence kit (ECL; Amersham Corp.) according to the manufacturer's instructions.

The phosphotyrosine-detected blots were then washed and incubated with 0.1 µg/ml S1CD in blocking solution with 25 µg/ml heparin for 2 h, washed 5 times with PBS containing 0.1% Nonidet P40 and then incubated with alkaline phosphatase-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories) in blocking solution containing 25 µg/ml heparin. Blots were washed 5 times with PBS containing 0.1% Nonidet P40 and then twice with alkaline phosphatase buffer (100 mM Tris, pH 9.5, containing 100 mM NaCl and 5 mM MgCl_2) and detected with Lumi-Phos® 530 according to the manufacturer's instructions (Boehringer-Mannheim, Indianapolis, IN, U.S.A.).

Phospholipase C- γ (PLC- γ) was detected on blots with an anti-PLC- γ monoclonal antibody (mAb) (Upstate Biotechnology

Inc., Lake Placid, NY, U.S.A.) with processing similar to the phosphotyrosine blots.

Phosphoamino acid analysis

NMuMG cells were incubated as described above with the exception that a low-phosphate (0.10 mM NaH_2PO_4) DMEM was used. Cells were prelabelled for 4 h with 0.75 mCi/ml $^{32}\text{PO}_4$ (DuPont–NEN, Boston, MA, U.S.A.) at 37 °C, then treated with pervanadate for 15 min, washed and treated with trypsin to generate the syndecan-1 C-terminal fragment. The fragment was immunoprecipitated with S1CD, subjected to PAGE and transferred to Zeta-Probe membrane. Radiolabelled protein was localized on the membrane by autoradiography, and the syndecan-1 C-terminal band was excised and incubated in 6 M HCl at 100 °C for 1.5 h. Samples were lyophilized and pellets resuspended in 20 μl of double-distilled water containing 1 mg/ml each unlabelled phosphoserine, phosphothreonine and phosphotyrosine (Sigma). A 2 μl aliquot of the sample was applied to a 0.25 mm cellulose TLC plate, and separated by electrophoresis at 1 kV for 1 h at 4 °C in pyridine/acetic acid/water (1:10:189, by vol.) (pH 3.5). The radiolabelled phosphoamino acids on the plate were identified by autoradiography and the phosphoamino acid standards were identified by spraying with 0.2 % ninhydrin in ethanol (Sigma).

Triton X-114 phase separation of cellular proteins

NMuMG cells were solubilized in PBS containing 1 % Triton X-114, 1 mM PMSF and 1 mM NEM at 4 °C for 10 min and then centrifuged at 5000 *g* for 10 min. An aliquot of the lysate was reserved for immunoprecipitation without further treatment and the remaining lysate was subjected to phase separation as described by Tiruppathi et al. [33]. After phase separation, Triton X-114 was added to a final concentration of 0.5 % to the aqueous phase and Tris-buffered saline (10 mM Tris/0.15 M NaCl, pH 7.4) was added to restore the original volume of the detergent phase; the samples were subsequently immunoprecipitated.

Quantitative analysis of medium and cell-associated syndecan-1

Cells were washed 4 times with 20 mM Hepes-buffered DMEM at 37 °C. Cells were incubated with this medium containing 0.1 % BSA with or without pervanadate for 30 min at 37 °C. Medium was removed and brought to 8 M urea. Cells were washed 5 times with PBS at 4 °C and extracted (2.5×10^6 cell equivalents/ml) in TUT (10 mM Tris, pH 8, 8 M urea, 0.1 % Triton X-100 and 1 mM Na_2SO_4) at 4 °C. Proteoglycans from cells and medium were isolated by incubation with DEAE-Sepharose (Pharmacia) for 1.5 h at 4 °C, and eluted in Microfuge spin columns with 100 mM Hepes, pH 6.5, containing 1 M NaCl, 10 mM CaCl_2 , 20 mM sodium acetate and 0.2 mg/ml BSA. Samples were brought to 0.3 M NaCl and serial dilutions in TBS were applied to Zeta-probe membrane equilibrated in TBS. The Zeta-probe membrane was incubated for 30 min with 0.05 % glutaraldehyde, washed in PBS and incubated for 2 h in blocking buffer containing 25 $\mu\text{g/ml}$ heparin at 37 °C. Blots were then incubated for 2 h with 1.0 $\mu\text{g/ml}$ mAb 281.2 in blocking buffer, washed 5 times with PBS and 0.1 % Nonidet P40, and then incubated with alkaline phosphatase-conjugated anti-rat antibody (Jackson ImmunoResearch Laboratories). The blots were washed 5 times in PBS, then once more in alkaline phosphatase buffer and detected using Lumi-Phos® 530 according to the manufacturer's directions.

RESULTS

Syndecan-1 is phosphorylated in vanadate-treated NMuMG cells

NMuMG cells express abundant levels of cell surface syndecan-1 [12]. The expression of this protein can be visualized via immunoblot analysis as intact proteoglycan, which migrates as a diffuse band, or as an enzymically denuded core protein, which migrates as a discrete band at 80 kDa. Alternatively, treatment of cells with mild trypsin rapidly cleaves the syndecan-1 core protein, leaving a cell-associated protein fragment consisting of the C-terminal cytoplasmic and transmembrane domains (C-terminal fragment). To isolate syndecan-1 C-terminal fragment, trypsin-treated cells were lysed and subjected to immunoprecipitation with an affinity-purified polyclonal antibody (S1CD) to the ten C-terminal amino acids of syndecan-1 (Figure 1A). The immunoprecipitated C-terminal fragment migrates as a 12 kDa fragment by gel electrophoresis. However, this is likely to be an inaccurate size assessment as the core protein, which migrates at over twice its calculated mass of 33 kDa, is notorious for anomalous migration.

The C-terminal fragment contains four tyrosines, with three exposed in the cytoplasmic domain (Figure 1A). To assess potential phosphorylation of these tyrosines, blots of S1CD immunoprecipitates were probed with mAbs specific for phosphotyrosine. However, this analysis failed to detect phosphorylated syndecan-1 (Figure 1B). In an attempt to retain phosphorylated tyrosines that may be dephosphorylated by phosphatases, NMuMG cells were pretreated with sodium orthovanadate, a general inhibitor of tyrosylphosphatases [34]. Treatment of cells for 1 h with orthovanadate results in significant syndecan-1 phosphorylation, as shown by the appearance of a tyrosine-phosphorylated band at 12 kDa in trypsin-treated cells (Figure 1B). The phosphorylation reaches a maximum by 3 h and is decreased by 6 h. Subsequent immunostaining of the blots with S1CD identifies the 12 kDa band as the C-terminal syndecan-1 fragment and demonstrates that this fragment is present on the blots at similar levels at all time points (Figure 1B). A faint band of a smaller product is consistently seen in the 1 and 3 h orthovanadate-treated samples and appears to represent a cleavage product of syndecan-1 which will be discussed in detail below.

Syndecan-1 is rapidly phosphorylated in pervanadate-treated NMuMG cells

The accumulation of phosphorylated syndecan-1 in orthovanadate-treated cells suggests either (i) that syndecan-1 is constitutively phosphorylated at low levels and that orthovanadate inhibits a phosphatase which rapidly dephosphorylates the protein or (ii) that the inhibition of cellular phosphatases leads to activation of a cytoplasmic kinase that recognizes and phosphorylates syndecan-1. In either case, the accumulation of phosphorylated protein is a slow process, suggesting that orthovanadate may be a relatively inefficient inhibitor of phosphatases under these conditions. To inhibit phosphatases more effectively and lead to the activation of intracellular tyrosine kinases, we used peroxides of vanadate that are formed through the addition of H_2O_2 to sodium orthovanadate (e.g. pervanadate) [28]. NMuMG cells pretreated with pervanadate (100 μM vanadate and 3 mM H_2O_2) for up to 30 min are viable but exhibit a more elongated shape (results not shown). Isolation of the syndecan-1 C-terminal fragment from pervanadate-treated cells provides several important results. First, cells treated with pervanadate cleave the C-terminal fragment into several products (Figure 2A). In addition to the 12 kDa product produced by trypsin

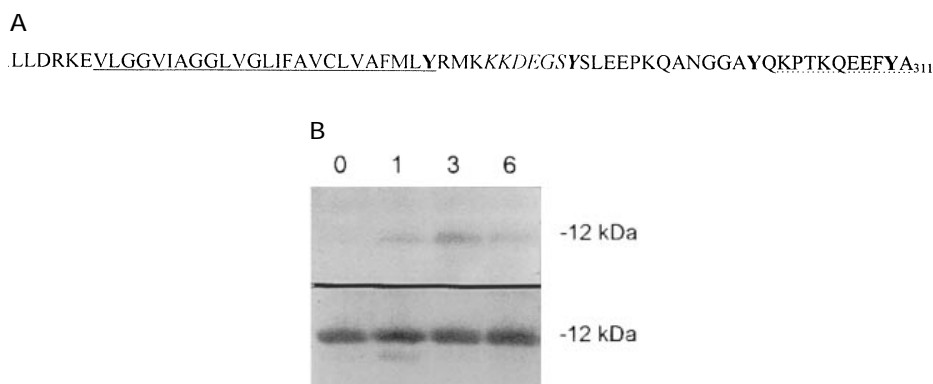


Figure 1 Phosphorylation of syndecan-1 cytoplasmic domain phosphorylation in orthovanadate

(A) The deduced amino acid sequence of the mouse syndecan-1 core protein [27] shows the four conserved tyrosines of the transmembrane/cytoplasmic domain in bold and a tyrosine kinase consensus phosphorylation sequence [25,26] adjacent to tyrosine-287 in italics. The transmembrane domain is underlined with a solid line and the recognition site for antibody S1CD is shown by a dashed line. (B) NMuMG cells were incubated with 1 mM sodium orthovanadate for 0, 1, 3 or 6 h at 37 °C, suspended by treatment with trypsin and then lysed in detergent. The C-terminal fragment of syndecan-1 was immunoprecipitated with S1CD, resolved by gel electrophoresis, transferred to Zeta-probe membrane and immunostained with either PY-20 (upper panel) or S1CD (lower panel).

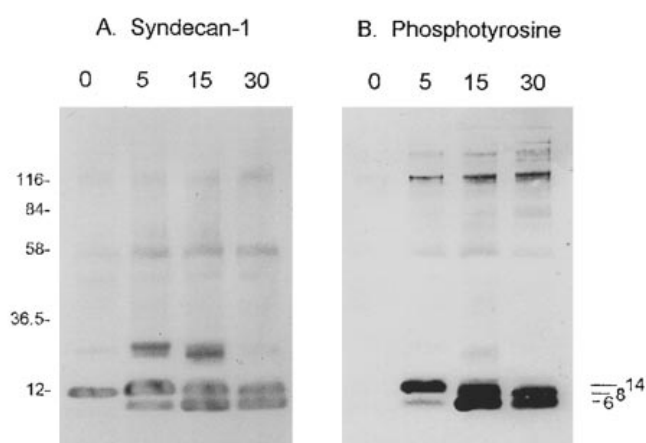


Figure 2 Time course of syndecan-1 cytoplasmic domain phosphorylation in pervanadate

NMuMG cells were treated with pervanadate (3 mM H_2O_2 and 0.1 M vanadate) for 0, 5, 15 or 30 min. S1CD immunoprecipitates from trypsin-treated cells were resolved by gel electrophoresis, transferred to Zeta-probe membrane and immunostained with (A) S1CD or (B) PY-20.

(seen as a single band lacking tyrosine phosphorylation at zero time), bands are resolved by SDS/PAGE at 6, 8 and 14 kDa, although the 8 and 14 kDa products are not found consistently in all experiments. These products are immunoreactive with S1CD antibody, indicating that they all retain the C-terminus of syndecan-1. At all time points, the syndecan-1 C-terminal fragment is present in equivalent amounts.

The second finding is that within 5 min of pervanadate addition at 37 °C, syndecan-1 is phosphorylated on tyrosine (Figure 2B), based on immunoreactivity with anti-phosphotyrosine antibody. Phosphorylation is detected primarily on the 6, 8 and 14 kDa bands. Tyrosine phosphorylation reaches a maximum within 15 min and is still prevalent at 30 min. Without pervanadate treatment at 37 °C, phosphorylation of syndecan-1 is not detected (Figure 2B). Phosphorylation does not occur at 4 °C with

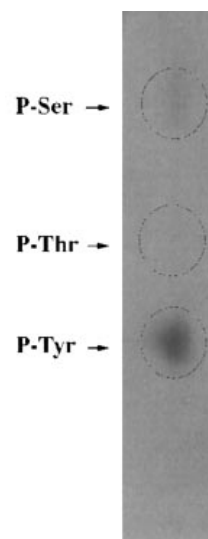


Figure 3 Phosphoamino acid analysis of syndecan-1 C-terminal fragment

S1CD immunoprecipitates of pervanadate-treated and trypsin-treated NMuMG cells labelled with $^{32}PO_4$ were resolved by gel electrophoresis and transferred to cationic membrane. The syndecan-1 C-terminal fragment on a membrane strip was hydrolysed and amino acids were separated by electrophoresis on a TLC plate. Radiolabelled amino acids were detected by autoradiography and compared with the positions of the phosphoamino acid standards as indicated by the dashed circles.

pervanadate, as all samples are treated with trypsin on ice in the presence of pervanadate for 10 min and only the cells previously incubated at 37 °C phosphorylate syndecan-1.

The diversity of bands may reflect phosphorylation and endogenous cleavage. The 14 kDa band probably represents the phosphorylated form of the C-terminal fragment generated by trypsin cleavage, which would otherwise be observed at 12 kDa. During longer pervanadate treatments, the 12 and 14 kDa bands disappear, apparently as the result of endogenous cleavage. In

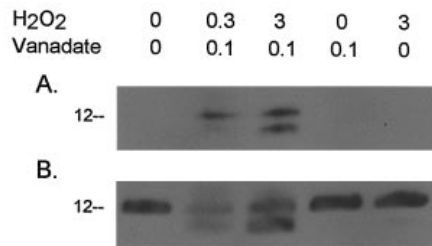


Figure 4 Both H₂O₂ and vanadate are required for syndecan-1 phosphorylation at short time points

NMuMG cells were treated with the indicated concentrations (mM) of vanadate and H₂O₂ for 15 min at 37 °C. S1CD immunoprecipitates from trypsin-treated cells were resolved by gel electrophoresis, transferred to Zeta-probe membrane and immunostained with (A) PY-20 or (B) S1CD.

concert with this endogenous cleavage, the 6 and 8 kDa bands become the major products, with the 8 kDa product possibly representing a hyperphosphorylated version of the 6 kDa protein. The ability to resolve these as distinct bands was variable in some experiments.

Phosphoamino acid analysis confirms that syndecan-1 is phosphorylated on tyrosine. Treatment of the cells with ³²PO₄ followed by a 15 min treatment with pervanadate and subsequent isolation of the syndecan-1 C-terminal fragment shows incorporation of label into 6–8 and 12 kDa bands that coincide with S1CD immunolocalization on blots (not shown). These labelled bands are not seen in the absence of S1CD immunoprecipitation. Excision of the combined 6–12 kDa bands and analysis of the hydrolysed protein by TLC demonstrates that the label is almost entirely in phosphotyrosine, although a small amount of phosphoserine is also detected (Figure 3).

Both H₂O₂ and vanadate are required for syndecan-1 phosphorylation at short time points

H₂O₂ alone can mimic some aspects of growth factor signalling [35,36]. To determine if the phosphorylation effects are due to H₂O₂ or to a combination of H₂O₂ and vanadate, cells were treated with H₂O₂ alone or with 0.1 mM vanadate together with increasing concentrations of H₂O₂ (Figure 4). There is no detectable phosphorylation of syndecan-1 upon treatment with vanadate or H₂O₂, or when there is no pretreatment (Figure 4). Syndecan-1 is tyrosine-phosphorylated when cells are treated with 0.1 mM vanadate and 0.3 mM H₂O₂ together, although treatment with 0.1 mM vanadate and 3 mM H₂O₂ together is more effective. Immunostaining with S1CD demonstrates that multiple phosphorylated syndecan-1 C-terminal fragments are precipitated (Figure 4B). The primary products are the 12 kDa product resulting from trypsin treatment and the 6–8 kDa fragments produced by endogenous cleavage. Treatment with H₂O₂ alone results in a small amount of the 6–8 kDa products, although no phosphorylation is detected. Vanadate alone or no treatment fails to produce cleavage products (Figure 4A).

Syndecan-1 transfected into Raji cells is phosphorylated

The multiple phosphorylated bands found in pervanadate-treated NMuMG cells all react with S1CD indicating that they contain the C-terminus of syndecan-1. However, NMuMG cells express other members of the syndecan family which contain highly homologous cytoplasmic domains. To verify that the multiple bands were all cleavage products of the syndecan-1 core protein,

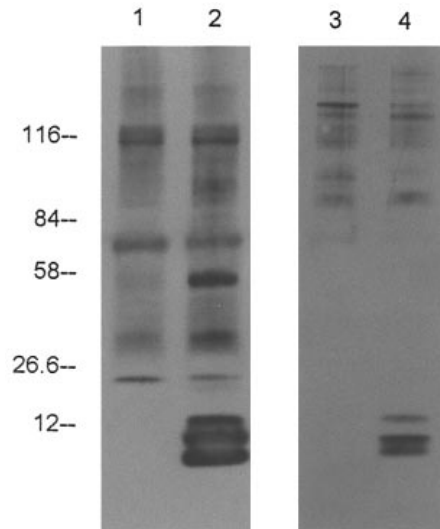


Figure 5 Tyrosine phosphorylation of syndecan-1 in Raji syndecan-1 transfectants

Raji parental cells (lanes 1 and 3) or Raji cells transfected with syndecan-1 (lanes 2 and 4) were pretreated with pervanadate for 15 min at 37 °C, treated with trypsin, and syndecan-1 C-terminal fragments were immunoprecipitated with S1CD. Immunoprecipitates were resolved by gel electrophoresis, transferred to Zeta-probe membrane and immunostained with S1CD (lanes 1 and 2) or PY-20 (lanes 3 and 4).

and not due to cross-reactivity with the other syndecans present in NMuMG cells, we examined either native Raji cells, which are syndecan-negative, or Raji S-1 cells that had been transfected with the cDNA for syndecan-1 and express syndecan-1 at the cell surface [31]. When Raji S-1 cells are treated with pervanadate followed by trypsin, three products are detected on immunoblots probed with S1CD, namely a 12 kDa product generated by trypsin, and two additional bands at 8 and 6 kDa (Figure 5). These bands are not present in the Raji parental cell line, indicating that all three bands are attributable to the transfected syndecan-1. As seen with the NMuMG cells, all three bands are also phosphorylated on tyrosine (Figure 5).

Phosphorylated syndecan-1 core protein is cleaved to yield low-molecular-mass products

The appearance of multiple phosphorylated C-terminal fragments of the syndecan-1 core protein demonstrates that pervanadate treatment activates a cellular protease and a kinase that recognize the core protein. This raises the question of whether or not both enzymes act on the native transmembrane proteoglycan. Upon treatment with heparitinase and chondroitin ABC lyase, the native syndecan-1 proteoglycan can be resolved on SDS/PAGE as a single core protein that migrates at 80 kDa (Figure 6A). After treatment of NMuMG cells with pervanadate, the intact core protein is indeed phosphorylated on tyrosine (Figure 6B). As expected, trypsin treatment of the cells results in the loss of both the 80 kDa phosphorylated band (Figure 6B) and the corresponding immunostained syndecan-1 core protein (Figure 6A), with the concomitant appearance of the phosphorylated 12 kDa C-terminal fragment and smaller products.

The smaller products seen in pervanadate-treated cells are also present in the absence of trypsin cleavage. When cells are treated with pervanadate, S1CD-reactive bands appear at 6 and 8 kDa, apparently produced by a cellular protease, with the additional

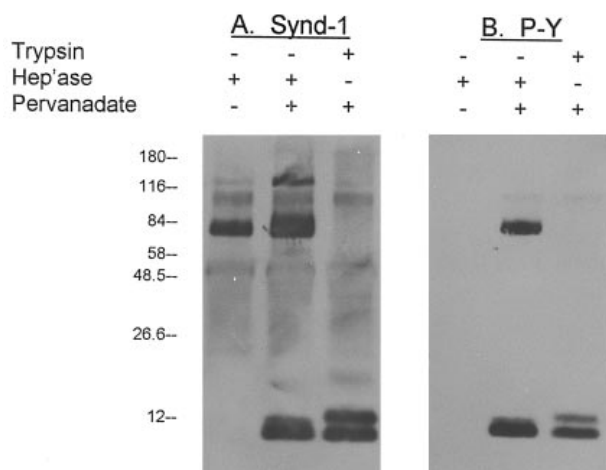


Figure 6 Tyrosine phosphorylation of the syndecan-1 core protein

NMuMG cells were incubated with or without pervanadate for 15 min at 37 °C, then shifted to 4 °C. These incubations were also combined with either treatment with heparitinase and chondroitin ABC lyase (Hep'ase) for 1 h at 37 °C or treatment with trypsin after the shift to 4 °C. Detergent lysates of the cells were immunoprecipitated with S1CD, resolved by gel electrophoresis, transferred to Zeta-probe membrane and immunostained with (A) S1CD or (B) PY-20.

12 kDa band appearing after trypsin treatment (Figure 6A). In cells treated with pervanadate the 6, 8 and 12 kDa products are all phosphorylated on tyrosine (Figure 6B). These data suggest that either the 6 and 8 kDa products are produced by a single cleavage and run differently as a result of the addition of multiple phosphates or more than one site is cleaved on syndecan-1 in response to pervanadate treatment. As the size of the 6 and 8 kDa products is not affected by treatment of the cells with trypsin, leading to yet smaller products, it is likely that the endogenous cleavage occurs closer to the C-terminus than the trypsin-recognition site.

Pervanadate treatment of NMuMG cells induces cleavage of most of the cell surface syndecan-1

To determine the effectiveness of pervanadate in inducing cleavage of syndecan-1, the core protein and its cleavage products were isolated from cells treated with pervanadate throughout a 30 min time course. The core protein is already diminished by 5 min of treatment and is greatly reduced by 30 min (Figure 7). There is a corresponding increase in the C-terminal fragments. At 5 min only the 6 kDa product can be detected and this product continues to increase until 15 min. The 8 kDa product can be detected by 15 min and continues to increase until 30 min.

The disappearance of the full-length core protein and subsequent appearance of lower-molecular-mass products may reflect degradation of proteoglycan that has been rapidly internalized, or it may represent a cell surface cleavage. These can be distinguished by monitoring the appearance of syndecan-1 ectodomain in the culture medium, which is indicative of a cell-surface-shedding mechanism. The addition of pervanadate for 30 min induces the release of syndecan-1 ectodomain into the medium (Figure 8), shown by isolating total proteoglycan from the medium and quantitatively analysing syndecan-1 on dot blots with mAb 281.2, a mAb to the ectodomain of syndecan-1. Untreated NMuMG cells shed only small amounts of syndecan-1 ectodomain during a 30 min incubation (Figure 8).

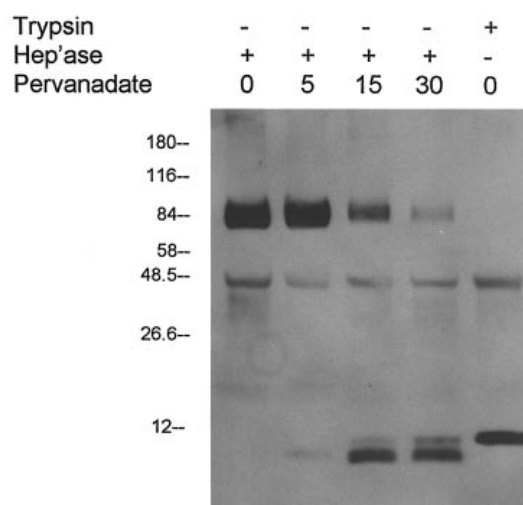


Figure 7 Pervanadate treatment cleaves syndecan-1 core protein on the cell surface and results in low-molecular-mass cleavage products

NMuMG cells treated with heparitinase and chondroitin ABC lyase (Hep'ase) were incubated with pervanadate for 0, 5, 15 or 30 min at 37 °C. Alternatively, cells were treated with trypsin without pervanadate incubation to generate the syndecan-1 C-terminal fragment. The cells were lysed in detergent and syndecan-1 C-terminal fragments were immunoprecipitated with S1CD. Immunoprecipitates were resolved by gel electrophoresis, transferred to Zeta-probe membrane and immunostained with S1CD.

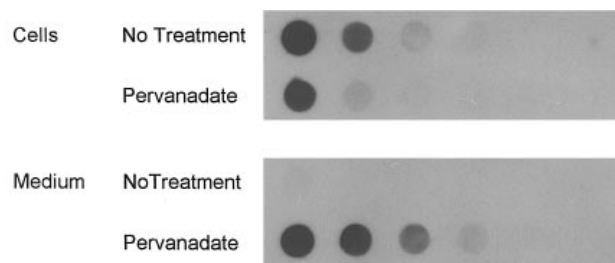


Figure 8 Syndecan-1 is shed into the medium of pervanadate-treated NMuMG cells

NMuMG cells were incubated with or without pervanadate (no treatment) for 30 min at 37 °C. Proteoglycan was isolated from the medium and cells on DEAE-Sephacel, then 3-fold dilutions of the eluate were applied to Zeta-probe membranes and immunostained with mAb 281.2.

The C-terminal fragment produced in pervanadate retains the hydrophobic membrane domain

The pervanadate-induced cleavage products migrate with much lower apparent molecular masses than the trypsin-generated fragment, indicating that they may be produced by intracellular cleavage of the cytoplasmic domain. A major difference between an intracellular and a cell surface cleavage would be retention of the transmembrane domain, the latter cleavage site producing a C-terminal fragment that retains hydrophobic properties. To test for hydrophobicity, the fragments were subjected to Triton X-114 phase separation in which hydrophobic membrane proteins are isolated in the detergent phase. As a control, PLC- γ , a cytoplasmic protein, was also isolated and partitioned. As expected, PLC- γ is isolated in the aqueous phase, which is typical of a non-membrane protein (Figure 9B). In contrast, both the

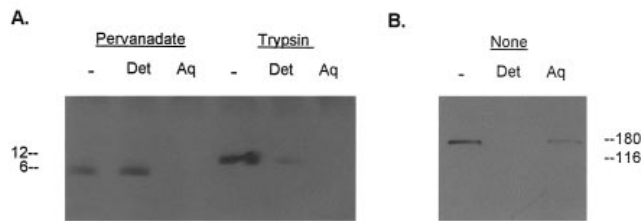


Figure 9 C-Terminal fragment generated by pervanadate-induced cleavage is hydrophobic

NMuMG cells treated with pervanadate or trypsin or untreated (none) were lysed in Triton X-114. An aliquot of the sample prior to separation (—), or equal aliquots of either the aqueous (Aq) or detergent (Det) phases after phase separation of the Triton X-114 were immunoprecipitated with S1CD (A) or anti-PLC- γ (B) and then resolved by gel electrophoresis, transferred to Zeta-probe membrane and immunostained with S1CD (A) or anti-PLC- γ (B).

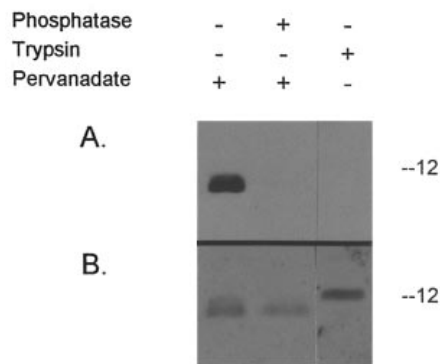


Figure 10 Phosphorylation of syndecan-1 C-terminal fragment retards its mobility in gel electrophoresis

S1CD immunoprecipitates from pervanadate-treated NMuMG cells were heated to 100 °C for 5 min and treated with or without potato acid phosphatase for 50 min at 32 °C. Immunoprecipitates were resolved by gel electrophoresis, transferred to Zeta-probe membrane and immunostained with (A) PY-20 or (B) S1CD. The C-terminal fragment derived by treatment of the cells with trypsin is shown for comparison.

6 kDa fragment produced by pervanadate treatment and the 12 kDa product of trypsin treatment separate into the detergent phase, indicating that they both retain at least part of the transmembrane domain (Figure 9A). As both are detected by S1CD, which recognizes the C-terminal 10 amino acids, this demonstrates that both fragments contain the complete cytoplasmic domain as well as most or all of the transmembrane domain.

Phosphorylation of syndecan-1 C-terminal fragment changes its migration during gel electrophoresis

To determine if phosphorylation affects the migration of these products, the isolated C-terminal fragments were treated with potato acid phosphatase to eliminate the phosphorylation. As seen previously, S1CD precipitates two proteins of molecular mass 8 and 6 kDa from pervanadate-treated cells (Figure 10B) that are phosphorylated (Figure 10A). Treatment with potato acid phosphatase eliminates the tyrosine phosphorylation of these proteins (Figure 10A). The dephosphorylated sample can be detected with S1CD staining as a single 6 kDa band (Figure 10B). It thus appears that the 8 kDa product co-migrates with the 6 kDa protein when it is dephosphorylated. As both proteins

are tyrosine phosphorylated, the 8 kDa band either contains multiple tyrosine phosphorylations or is phosphorylated on serine in addition to tyrosine. Similarly, the 14 kDa product seen occasionally in pervanadate-treated cells after trypsinization is most likely a more highly phosphorylated version of the 12 kDa trypsin-cleavage product.

DISCUSSION

Syndecan-1 is phosphorylated on tyrosine in cells treated with pervanadate, demonstrating that the syndecan-1 cytoplasmic domain is a substrate for an intracellular kinase, presumably part of an intracellular signal-transduction pathway. Syndecan-1 has three conserved tyrosines in the cytoplasmic domain [1,27], one of which (Y²⁸⁷) lies in a consensus sequence for recognition by a tyrosine kinase [25,26]. It is not known at present which, or if more than one, of the tyrosines is phosphorylated. Concurrent with the phosphorylation, although potentially as a separate response to the activation of intracellular kinases, syndecan-1 is cleaved into two components: the ectodomain is shed into the medium and the cytoplasmic domain with all or part of the transmembrane domain is retained with the cell. Although phosphorylation of the cytoplasmic domain is detected prior to shedding, it is not known whether it stimulates the shedding process.

Kinases that rely on auto- or trans-phosphorylation for activation are also inactivated by phosphatases that remove these phosphates. Thus vanadate inhibition of phosphatases would be predicted to release negative controls imposed on cellular kinases and stimulate signal-transduction cascades in the cell [29]. Indeed, orthovanadate and pervanadate have been used previously in this manner to stimulate cellular responses that are dependent upon phosphorylation cascades [29,37–41]. The effect of pervanadate in the NMuMG cells may largely reflect this function, together with enhanced retention of the phosphates during treatment and protein isolation. The more rapid effect of pervanadate compared with orthovanadate is probably due to its greater efficacy as a phosphatase inhibitor, although the vanadates may also have differing specificities [40].

The identity of the kinase that phosphorylates syndecan-1 is not known. It is postulated to be a tyrosine kinase that is regulated by phosphorylation. As syndecan-1 is not readily isolated in a phosphorylated form in the absence of pervanadate, the kinase is not highly active in standard cell culture conditions. An alternative is that the kinase is constitutively active and that pervanadate blocks the rapid phosphate removal by an intracellular phosphatase. This seems unlikely on the basis of current knowledge of kinases in signal-transduction systems, where kinase activity is believed to be transient. However, it cannot be ruled out that the accumulation of phosphorylated syndecan-1 over a period of hours during orthovanadate treatment may reflect the accumulation of constitutively phosphorylated syndecan-1 which is normally subjected to rapid dephosphorylation by a vanadate-sensitive phosphatase.

Phosphorylation is an important regulator of cellular function for other cell surface receptors that do not contain inherent kinase activity, such as the integrins and the cadherins [42,43]. Members of the integrin family can be phosphorylated, and binding of their ligands induces phosphorylation of intracellular proteins including focal adhesion kinase [16] and paxillin [44]. Phosphorylation of catenins by transfection of cells with *v-src* abolishes their interaction with cadherins and leads to changes in cellular morphology [45]. Similarly, phosphorylation of syndecan-1 may alter its interaction with other cellular proteins. The phosphotyrosine may provide a binding site for the SH2 domains

of regulatory proteins, thus participating in the assembly of an adhesive signalling complex, or may alter its anchorage to the cytoskeleton. Clustering of syndecan-1 using antibodies, thus mimicking its aggregation into adhesive sites, enhances its retention in the Triton-insoluble cytoskeletal residue and causes it to align with actin filaments [11,12]. This alignment is dependent on the presence of the cytoplasmic domain [11].

In addition to stimulating phosphorylation of syndecan-1, pervanadate treatment induces cleavage of the proteoglycan and the release of its ectodomain into the culture medium. This release of syndecan-1 allows rapid changes in cell surface expression and may be a means of changing the cellular response to heparan sulphate-binding ligands such as fibroblast growth factors or extracellular matrix ligands. Extracellular proteolytic cleavage of proteins is a common mechanism of altering cell surface expression of a wide variety of molecules and is typically stimulated by treatment with phorbol esters [e.g. phorbol 12-myristate 13-acetate (PMA)], as shown for L-selectin [46], tumour necrosis factor α receptor [47,48], amyloid precursor protein [49,50], colony-stimulating factor-1 (CSF-1) [51] and CSF-1 receptor [52].

PMA, an activator of the serine/threonine protein kinase C (PKC), has been shown to stimulate release of proteoglycans from the cell surface concurrent with changes in cellular morphology and cell proliferation [53]. Similarly, we find that the shedding of syndecan-1 is promoted by PMA and that the PMA-induced shedding is reduced by treatment with staurosporine, an inhibitor of PKC (J. Reiland, V. L. Ott, C. S. Lebakken, C. Yeaman, J. McCarthy and A. C. Rapraeger, unpublished work). Treatment of NMuMG cells with 200 nM PMA generates the 6 kDa C-terminal cleavage fragment, although no tyrosine phosphorylation is detected (V. L. Ott and A. C. Rapraeger, unpublished work). This suggests that one mechanism by which pervanadate may induce shedding is by activation of PKC. This is unlikely to be a direct effect as PKC activity is not known to be controlled by phosphorylation. Also, as PKC is a serine/threonine kinase, it cannot be directly responsible for the tyrosine phosphorylation of syndecan-1. Furthermore, as we fail to detect syndecan-1 tyrosine phosphorylation in response to PMA treatment, the kinase that phosphorylates syndecan-1 does not appear to be downstream from PKC. It is a possibility, however, that activation of a kinase upstream from PKC leads to both PKC activation and syndecan-1 phosphorylation on tyrosine. Shedding of syndecan-1 also occurs in response to other stimuli, suggesting that they also may act through PKC. It is rapidly shed from the cell surface when cells are treated with EDTA to promote detachment from their substratum [54]. Transformed mouse keratinocytes expressing high levels of Ha-ras p21 display increased shedding of syndecan-1 [55]. Also, NIH-3T3 cells release syndecan-1 into the medium when treated with both transforming growth factor β and basic fibroblast growth factor [56].

The current data do not demonstrate that syndecan-1 phosphorylation is a prerequisite for the shedding process to occur, although they do occur in the same time frame. It is perhaps more likely that the shedding occurs as a separate pathway that activates a membrane protease. The tumour necrosis factor α receptor and B-amyloid precursor protein, examples of proteins that are phosphorylated and cleaved in response to PMA treatment, are cleaved even when phosphorylation is blocked by mutation of the cytoplasmic tyrosines and serines [57,58]. Mutation of the serines and tyrosines in the syndecan-1 cytoplasmic domain will be required to address whether phosphorylation of syndecan-1 itself is required for shedding.

The intracellular kinases that are activated by pervanadate

and lead to phosphorylation and cleavage of syndecan-1 are not known at present. Their activation may occur through signalling of either growth factors or adhesive events; changes in the latter are known to affect syndecan-1 cleavage [54]. This is likely to provide a mechanism whereby the participation of the heparan sulphate proteoglycan in cell attachment, migration and response to heparan sulphate-binding growth factors is regulated.

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